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ORIGINAL ARTICLE

Molecular Characterization of *Thyroid Hormone Receptors* (*TRs*) and their Responsiveness to T3 in *Microhyla fissipes*

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Abstract To explore and enrich the molecular mechanisms of *thyroid hormone receptors* (*TRs*) in the metamorphosis of amphibians, the cDNA sequences of *TRα* and *TRβ* in *Microhyla fissipes* were cloned and characterized. *TRα* was 1 706 bp in length with an open reading frame (ORF) of 1 257 bp encoding a predicted protein of 418 amino acids and *TRβ* was 1 422 bp with an ORF of 1 122 bp encoding a predicted protein of 373 amino acids. Their protein sequences contained 4 conserved domains of the nuclear receptor superfamily with two highly conserved cysteine-rich zinc fingers in the DNA-binding domain, whereas TRβ was 42 amino acids shorter in its A/B domain than TRα. Highly-conserved sequences and structures indicated their conserved functions during metamorphosis. *TRα* expression reached peak at 12 h and then decreased from 12 h to 48 h. While dramatically up-regulated *TRβ* was observed after exposure of T3 within 24 h, and it was down-regulated from 24 h to 48 h. The expression pattern of *TRβ* is similar to that in the natural metamorphosis. Furthermore, tadpoles treated 24 h also resembled the climax of metamorphosis tadpoles and *TRβ* expression had higher responsiveness than *TRα* to T3 in *M. fissipes*. These results suggest *M. fissipes* may serve as the model to assay environmental compounds on TH signaling disruption.

Keywords *Microhyla fissipes*, *thyroid hormone receptors*, functional characteristic, expression pattern, responsiveness

1. Introduction

Amphibian metamorphosis is a complex process, in which various organs and tissues undergo dramatically remodeling to transform from larva to juvenile (Atkinson *et al.*, 1998; Brown and Cai, 2007; Du *et al.*, 2017). Although complex, this development process is completely initiated and orchestrated by only one hormone, thyroid hormone (TH) (Buchholz *et al.*, 2006). The TH signaling pathway in amphibians has been well studied: when TH is absent, the unliganded TR/9-cis-retinoic acid receptor (RXR) heterodimers recruit corepressors to repress the transcription of downstream target genes; once liganded with TH, the TR/RXR heterodimers undergo conformational change and thus allow to recruit coactivators to activate the same group of downstream genes (Morvan-Dubois *et al.*, 2008; Grimaldi *et al.*, 2013; Zhao *et al.*, 2016). Therefore, *TRs* play important roles in the metamorphosis by acting as ligand-dependent transcriptional factors.

TRs, members of a large superfamily of nuclear receptors (NR), possess a similar domain structure as that found in the other NRs: an N-terminal A/B domain with binding sites for transcriptional coregulators, a central DNA-binding domain C (DBD) containing two "zinc fingers" for target gene recognition, a D domain (hinge region) containing the nuclear localization signal, and a C-terminal ligand-binding domain E/F (LBD) where thyroid hormone binds and activates the receptor (Chen *et al.*, 2014). There are two closely related families of *TRs* called *TRα* and *TRβ* in vertebrate

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(Yaoita *et al.*, 1990; Chen *et al.*, 2014). *TRα* and *TRβ* are differentially expressed in various tissues of different species (Kawahara *et al.*, 1991). Particularly, the *TRα* mRNA increases throughout the premetamorphosis stage of tadpole development, and falls after the climax of metamorphosis to a lower level in frogs (Yaoita and Brown, 1990). The *TRβ* mRNA is barely detectable during premetamorphosis. In synchrony with the onset of endogenous TH synthesis by the thyroid gland, the level of *TRβ* mRNA rises in parallel with endogenous TH, reaching a peak at the climax of metamorphosis and drops after metamorphosis (Choi *et al.*, 2015). Although TH signaling pathway has been well studied, functional of *TRα* and *TRβ* during metamorphosis have not been clearly characterized. *TRα*-deficient tadpoles developed faster with smaller body size than their wild-type siblings suggesting that *TRα* played important roles in controlling the timing of *Xenopus tropicalis* metamorphosis (Choi *et al.*, 2015; Wen and Shi, 2015). Furthermore, disrupted *TRα* had different effect on the development of larval and juveniles and the metamorphosis of different organs (Choi *et al.*, 2017, Wen *et al.*, 2017). Different from *TRα*-knockout tadpoles, significantly delayed tail regression, the reduction in olfactory nerve length and head narrowing by gill absorption were detected in *TRβ*-knockout tadpoles (Nakajima *et al.*, 2017). The different relative abundance levels of *TRα* and *TRβ* transcripts induced by T3 where the general pattern was *TRα* $\geqslant$ *TRβ* in *R. catesbeiana*, while *TRα* $\leqslant$ *TRβ* in *Xenopus laevis* (Veldhoen *et al.*, 2014). *TRβ* was highly expressed during metamorphosis in *M. fissipes* and *X. laevis*, but *TRα* showed especially low expression in *M. fissipe*s, implying that *TRβ* is essential for initiating metamorphosis, at least in *M. fissipes* (Zhao *et al.*, 2016).

Microhyla fissipes is a small-sized anuran from the family Microhylidae suborder Neobatrachia (Figure 1). Due to the special expression pattern of *TRs* in *M. fissipes* mentioned above, it is important to clone *TRs* and understand the molecular mechanism of them in regulating metamorphosis. Furthermore, because of its characteristics (including wide distribution, fast development, development *in vitro*, strong survivability, biphasic life cycle, small body size, diploid and transparent tadpoles) and being induced to metamorphose by exogenous TH, *M. fissipes* may be an ideal model to evaluate the possible effects of environmental compounds on the thyroid system (Liu *et al.*, 2016).

Therefore, it is necessary to characterize *TRs* and evaluate responsiveness to TRs agonist (such as 3,3',5-Triiodo-L-thyronine, T3) in *M. fissipes*.

Figure 1 Photograph of *Microhyla fissipes*.

In this study, we have isolated, characterized, and phylogenetically analyzed *TRα* and *TRβ* gene in *M. fissipes*, examined their expression pattern after T3 treatment and explored the utility of *M. fissipes* as a model species for assaying TH signaling disrupting effects.

2. Materials and Methods

2.1. Animals sampling and experimental treatments

Mature female and male *M. fissipes* were collected from Shuangliu, Chengdu, China (30.5825° N, 103.8438° E) in June, 2016. The male and female were injected luteinizing hormone-releasing hormone a (LHRHa) with 0.3 μg/g body weight resolving dosage. Fertilized eggs were obtained from one pair of frogs and incubated in the dechlorinated tap water. Five days later, tadpoles were fed with spirulina powder once daily and subjected to a 12:12 h light:dark cycle at 25 ± 0.6°C. The developmental stage of tadpole was recorded using the *M. fissipes* developmental table (Wang *et al.*, 2017). Tadpoles at stage 40 (metamorphosis climax) were selected for gene cloning. Tadpoles of stage 33 (premetamorphosis, oar-shaped limb bud) were selected to treat with 10 nmol/L 3,3',5-triiodo-l-thyronine sodium (T3, Sigma-Aldrich, USA) for 48 h. The chemicals were renewed after 12 h of exposure when the medium was also refreshed. Tadpoles treated for 0 h, 12 h, 24 h, 36 h, and 48 h were collected for quantitative real-time (RT) PCR (n = 3 for each time point). Tadpoles treated 0 h were set as the control group. After anesthetization by MS222, tadpole sample was frozen immediately in liquid nitrogen, and then stored at –80°C for RNA extraction.

The care and treatment of animals in this study were performed according to the Guideline for the Care and Use of Laboratory Animals in China. The animal experiments were approved by the Experimental Animal

Use Ethics Committee of the Chengdu Institute of Biology (Permit Number: 2016036).

2.2. Cloning and molecular characterization of *TRs* Total RNA was extracted using TransZol (Transgen Biotech, Beijing, China), following the manufacturer's instructions. Total RNA concentration was calculated using Nanodrop ND-1000 (Nanodrop, DW, USA). Partial cDNA sequence of *TRs* (*TRα*: comp77374_c0 ; *TRβ*: comp124487_c3) were obtained from *M. fissipes* transcriptome (Zhao *et al.*, 2016). According to the partial cDNA sequence, two specific primers for each gene, GSP3-1 and GSP3-2 (Table 1) were designed to amplify the 3' terminal regions by nested PCR. The 3' DNA ends were obtained using the SMART RACE cDNA Amplification Kit (Clontech, CA, USA) in accordance with the manufacturer's instructions. Products of rapid amplification of cDNA ends (RACE) were cloned into pMD 18-T vector (TaKaRa, Japan) and sequenced using an automated DNA sequencer ABI3730 (Thermo Fisher Scientific, CA, USA) by Sangon Biotech Co. Ltd. (Shanghai, China).

The amino acid sequence was deduced from the coding region via DNAStar (version 6.13). The cDNA sequence and the deduced amino acid sequence were analyzed using BLASTN and BLASTP, respectively. Deduced amino acid sequences of amphibian were aligned for analysis of putative conserved functional residues by Clustal X. The relevant amino acid sequences were obtained from the NCBI GenBank database: *Rugosa rugose TRα* BAM15695.1, *Pelophylax nigromaculatus TRα* AGT55994.1, *Rana chensinensis TRα* AIA98429.1, *X. laevis TRαA* NP_001081595.1, *X. laevis TRαB* BAL70322.1, *X. tropicalis TRα* NP_001039261.1, *X. tropicalis TRβ* NP_001039270.1, *X. laevis TRβA* NP_001090182.1, *X. laevis TRβB* NP_001081250.1, *P. nigromaculatus TRβ* AGT55995.1, *R. chensinensis TRβ* AIA98430.1.

Multiple cDNA sequences of *TRα* and *TRβ* from 17 species represented vertebrate (Mammalia, Aves, Reptilia, Amphibia, Pisces) and invertebrate were used in the sequence alignment by Clustal X. A phylogenetic tree was constructed by using the maximum likelihood (ML) method with the MEGA 6 (Tamura *et al.*, 2013), and the reliability of the tree was assessed by the bootstrap method with 1,000 replications. The gene accession numbers are: *Alligator mississippiensis TRα* NM_001287278.1, *Branchiostoma lanceolatum TRα* EF672345.1, *Crassostrea gigas TRα* KP271450.1, *Gallus gallus TRα* NM_205313.1, *Homo sapiens TRα* AB307686.1, *Oryzias latipes TRα* AB114860.1, *Rattus norvegicus TRα* M18028.1, *P. nigromaculatus TRα* KC139354.1, *R. catesbeiana TRα* L06064.1, *R. chensinensis TRα* KJ579109.1, *R. rugose TRα* AB683466.1, *Schistosoma mansoni TRα* AY395038.1, *S. mansoni TRβ* AY395039.1, *X. laevis TRαA* M35343.1, *X. laevis TRαB* AB669465.1, *X. tropicalis TRα* NM_001045796.1, *Nanorana parkeri TRα* XM_018569566.1, *Oryzias latipes TRα* NM_001104705.1, *Danio rerio TRα* NM_131396.1, *R. rugosa TRβ* AB683467.1, *R. chensinensis TRβ* KJ579110.1, *R. catesbeiana TRβ* L27344.1, *P. nigromaculatus TRβ* KC139355.1, *N. parkeri TRβ* XM_018570223.1, *X. laevis TRβA* NM_001096713.1, *X. laevis TRβB* NM_001087781.1, *X. tropicalis TRβ* AB244214.1, *G. gallus TRβ* NM_205447.2, *A. mississippiensis TRβ* NM_001287311.2, *H. sapiens TRβ* M26747.1, *R. norvegicus TRβ* J03933.1, *O. latipes TRβ* AB114861.1, *D. rerio TRβ* NM_131340.1.

2.3. RNA isolation and gene expression analysis For *TRs* mRNA expression analysis after exposure of T3, we conducted qRT-PCR. Total RNA was extracted from tadpoles with TranZol reagent and first strand cDNA was synthesized from the same amount of RNA (1 μg) for each sample via TranScript All-in-One First-Strand cDNA Synthesis SuperMix for qPCR (TransGen, Beijing, China) with oligo (dT) primer. The expression of *TRα* and *TRβ* mRNA was analyzed by qRT-PCR with the primer *TRα* and *TRβ* (Table 1). *Rpl37* gene was used as a reference gene to normalize mRNA expression of *TRs*. PCR was performed in a reaction volume of 20 μl containing 10 μl of *TransStart*® Tip Green qPCR SuperMix (2×), 0.4 μl of Passive Reference Dye (50×), 0.4 μl each of forward and reverse primer (10 μmol/L), 8.2 μl of ddH_2O, and 1 μl of cDNA. Amplification was carried out in 7300plus (ABI, CA, USA), including 5 min at 95°C and 45 cycles of 5 s at 95°C and 31 s at 60°C, followed by a melting curve analysis. Each sample was run in triplicates. Each reaction was verified to contain a single product of the correct size by agarose gel electrophoresis. Quantitative data were shown as mean ± SD (n = 3). The fold change of *TRs* expression after T3 treatment was determined by $2^{-\Delta\Delta Ct}$ (cycle threshold, Ct). Data were then subjected to one-way analysis of variance (ANOVA) with SPSS Statistics 13.0 (SPSS Inc., Chicago, IL, USA). $P < 0.05$ was regarded as statistically significant.

3. Results

3.1. Molecular characterization of *TRs* in *M. fissipes* The full-length cDNA sequences of *TRα* and *TRβ* were obtained by RNA-seq and 3'-RACE strategies. The full-

Table 1 Primers used in this study. F and R denote forward and reverse primer, respectively.

Funticon	Primers	Sequence (5' to 3')	Size (bp)	Tm (°C)
Cloning	*TRαGSP3-1*	CCATCGCAAACACAACATTCCCCA	621	50
	TRαGSP3-2	TGACTTGCGTATGATCGGAGCCTG	567	60
	TRβGSP3-1	GCTGATGAAAGTCACCGACCTCCG	424	50
	TRβGSP3-2	CGCCAGCAGGTTCTTGCACATG	386	60
	3′ CDS primer A	AAGCAGTGGTATCAACGCAGACTAC(T) 30 VN(N = A, C, G, or T; V = A, G, or C)		
	UPM	CTAATACGACTCACTATAGGGCAAGCAGTGGTATCAACGCAGAGT CTAATACGACTCACTATAGGGC		
RT-PCR	*TRα*	F: GCAGCCGTGCGCTATGAT R: TGCCATCTCACCGCTTAGTG	57	60
	TRβ	F: TGGCCAAAACTGCTGATGAA R: GCGTGGCAGGCTCCAA	56	60
	Rpl37	F: CCAAAAAGCGCAACAACCA R: TTGCGAATCTGACGGACTTG	59	60

length of *TRα* cDNA was 1 706 bp in length and contained an open reading frame (ORF) of 1 257 bp, which encoded a peptide of 418 amino acids (Figure 2a). The *TRβ* cDNA was 1 422 bp with an ORF of 1 122 bp, which encoded a peptide of 373 amino acids (Figure 2b). And the 3' untranslated region of *TRα* and *TRβ* were 449 bp and 300 bp, respectively. Two sequences were submitted to the GenBank (GenBank accession numbers: MG596879 and MG596880). The homologies of nucleotide sequences and deduced amino acid sequences between *TRα* and *TRβ* in *M. fissipes* were 61% and 72%, respectively. The calculated molecular weight of TRα polypeptide was 47.7 kDa, and the theoretical isoelectric point (pI) was 7.08, while the calculated molecular weight of TRβ polypeptide was 42.4 kDa with pI 6.76.

A multiple alignment of *TRs* deduced amino acid sequences was performed in amphibians (Figure 3). The deduced amino acids of both TRα and TRβ were composed of the N-terminal hypervariable region (A/B domain), a conserved DNA-binding domain (DBD domain), a hinge region (D domain), and a ligand-binding domain (LBD domain). TRα and TRβ in *M. fissipes* had high similarity in the DBD, D and LBD domains, whereas there was a deletion of 42 amino acids in TRβ compared with TRα in the A/B domain (Figure 3). These deletion between TRα and TRβ in *M. fissipes* corresponded to the differences found in *X. laevis*, *X. tropicalis*, *R. chensinensis* and *P. nigromaculatus*. Although there were several different sites in the A/B domain among TRs, the N-terminal signature sequence (NTSS) was well conserved. The conserved cysteine residues that comprise the two zinc fingers and the regulatory elements P-box as well as the T-box and A-box in the DBD were conserved. Furthermore, the consensus motif I (spanning helix 3–6) and motif II (from helix 8 to helix 10) and the putative AF2 activation domain core (helix 12) were also identified. Therefore, this highly-conserved feature is likely to indicate pivotal significance of *TRs* in thyroid signaling pathway.

3.2. Evolutionary relationships of TRs BLASTP and BLASTN in NCBI indicated that *TRα* and *TRβ* shared different levels of homology with other species. The amino acid sequences of *M. fissipes TRα* had highest homolgy with *R. rugosa*, *N. parkeri*, *R. chensinensis* (98%, 10 different sites out of 418 amino acids), while *M. fissipes TRβ* had highest homolgy with *R. catesbeiana* (98%, 8 different sites out of 373 amino acids). Nucleotide sequences of *M. fissipes TRα* and *TRβ* shared high homology with *N. parkeri* (92% and 93%), *P. nigromaculatus* (92% and 92%), *R. rugosa* (93% and 92%), *X. tropicalis* (88% and 87%), *X. laevis A* (88% and 80%), *X. laevis B* (88% and 78%) and had a lower homology with *G. gallus* (65% and 74%), *A. mississippiensis* (63% and 63%), *H. sapiens* (58% and 47%), *D. rerio* (57% and 49%), *B. lanceolatum* (47% and 49%), respectively.

A phylogenetic tree constructed by the ML method from a multiple alignment of nucleotide sequences of *M. fissipes TRα* and *TRβ* and a wide range of counterparts in various species including invertebrate, reptiles, birds, mammals and other amphibian (Figure 4). The phylogenetic tree showed that *TRα* and *TRβ* grouped into two highly consistent and separate clades. In both *TRα* and *TRβ* clades, *TRs* from *M. fissipes* have similar positions in the phylogenetic tree. Furthermore, phylogenetic tree constructed based on *TRs* cDNA sequences was consistent well with the taxonomic positions of these organisms.

3.3. Responsiveness of *M. fissipes TRs* expression to exogenous T3 In the presence of exogenous T3,

a.

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ATGGACCAGAATCTTAGCGGGCTGGACTGCTTGTCAGAGCCAGATGAAAAAAGGTGG      57
 M  D  Q  N  L  S  G  L  D  C  L  S  E  P  D  E  K  R  W
CCGGATGGGAAGCGTAAAAGAAAGAACAGCCAATGCATGGGAAAAAGCGGCATGTCA     114
 P  D  G  K  R  K  R  K  N  S  Q  C  M  G  K  S  G  M  S
GGTGACCGTTCGGTGTCTCTGCTCTCTGCAGGGTACATCCCTAGCTACCTGGACAAAGAT  174
 G  D  R  S  V  S  L  L  S  A  G  Y  I  P  S  Y  L  D  K  D
GAGCCGTGCGTGGTGTGCAGTGACAAGGCCACAGGGTACCACTACCGATGTATTACC     231
 E  P  C  V  V  C  S  D  K  A  T  G  Y  H  Y  R  C  I  T
TGTGAGGGTTGCAAGGGCTTCTTCCGTCGAACCATCCAGAAGAACCTGCACCCTTCC     288
 C  E  G  C  K  G  F  F  R  R  T  I  Q  K  N  L  H  P  S
TACTCCTGCAAGTATGATGGCTGCTGCATCATCGATAAGATCACCCGGAATCAGTGCCAG  348
 Y  S  C  K  Y  D  G  C  C  I  I  D  K  I  T  R  N  Q  C  Q
CTCTGCCGATTCAAGAAATGTATCGCAGTGGGCATGGCAATGGACCTTGTCCTGGATGAT  408
 L  C  R  F  K  K  C  I  A  V  G  M  A  M  D  L  V  L  D  D
TCCAAGCGGGTAGCCAAGCGGAAGCTGATTGAGGAAAATCGGGAACGGCGTCGGAAG     465
 S  K  R  V  A  K  R  K  L  I  E  E  N  R  E  R  R  R  K
GAGGAGATGATGAAGTCTCTGCAGCAGCGACCTGAGCCAAGCAGCGAGGAATGGGAG     522
 E  E  M  M  K  S  L  Q  Q  R  P  E  P  S  S  E  E  W  E
CTGATCCGCATAGTGACAGAAGCTCACCGGAGCACCAACGCACAGGGCAGCCACTGG     579
 L  I  R  I  V  T  E  A  H  R  S  T  N  A  Q  G  S  H  W
AAGCAGCGCAGGAAGTTCTTGCCGGATGACATTGGGCAGTCTCCGATGGCCTCCATGCCA  639
 K  Q  R  R  K  F  L  P  D  D  I  G  Q  S  P  M  A  S  M  P
GATGGTGATAAAGTAGACCTGGAGGCGTTCAGTGAGTTCACCAAAATCATCACCCCTGCA  699
 D  G  D  K  V  D  L  E  A  F  S  E  F  T  K  I  I  T  P  A
ATCACCCGAGTGGTGGACTTCGCCAAGAAGCTGCCCATGTTCTCTGAGCTGCCCTGTGAA  759
 I  T  R  V  V  D  F  A  K  K  L  P  M  F  S  E  L  P  C  E
GACCAGATCATCCTGTTGAAAGGATGCTGTATGGAAATTATGTCTCTTCGGGCAGCCGTG  819
 D  Q  I  I  L  L  K  G  C  C  M  E  I  M  S  L  R  A  A  V
CGCTATGATCCGGACAGCGAAACTCTGACACTAAGCGGTGAGATGGCAGTGAAGCAA     876
 R  Y  D  P  D  S  E  T  L  T  L  S  G  E  M  A  V  K  Q
GAACAGCTGAAGAATGGAGGGCTGGGGGTTGTCTCTGATGCTATATTTGACTTGGGAAGA  936
 E  Q  L  K  N  G  G  L  G  V  V  S  D  A  I  F  D  L  G  R
TCTTTGTCTGCGTTCAATCTGGATGACACAGAAGTGGCCCTGCTGCAGGCTGTCCTGCTC  996
 S  L  S  A  F  N  L  D  D  T  E  V  A  L  L  Q  A  V  L  L
ATGTCCTCAGACCGGACGGGTTTGATCTGCACGGACAAAATAGAGAAATGTCAGGAG    1053
 M  S  S  D  R  T  G  L  I  C  T  D  K  I  E  K  C  Q  E
ACGTACCTCCTCGCCTTCGAACACTACATCAACCATCGCAAACACAACATTCCCCACTTC  1113
 T  Y  L  L  A  F  E  H  Y  I  N  H  R  K  H  N  I  P  H  F
TGGCCCAAGCTCCTTATGAAAGTGACTGACTTGCGTATGATCGGAGCCTGCCATGCCAGT  1173
 W  P  K  L  L  M  K  V  T  D  L  R  M  I  G  A  C  H  A  S
CGCTTCCTGCACATGAAGGTTGAGTGCCCCACAGAGCTCTTCCCGCCTCTCTTCCTTGAG  1233
 R  F  L  H  M  K  V  E  C  P  T  E  L  F  P  P  L  F  L  E
GTGTTTGAGGACCAGGAAGTC TGA GGGGCAGTGGAGTGGGCCAGAGACTAAAGGTAAT  1291
 V  F  E  D  Q  E  V  -
TTTGCTTGGCTCTTTCCAGCCTGCCAACCCTTACAAGGCTCTTGTGTCCTGCGAATGATG  1349
CAAGGGAGTGGCGGGATAGGTCCTTCTGATAATGAAAACTGCCTTACGTAGTTGCAATG   1408
CCTGGCCTTCCTGCTGCCTGATTTCTAACCAAGCTGGAGAGAGTTGAAGCTTGCCACAG   1467
CGCCTATCCTTCAGCAAAACTGTCCTTTTTGACAGCATTAGTAAAGCACAGTGGTACTTA  1527
AAGCAATAGATTCCATCTGGGCTCTTGTCGAGTCACTGTATGTAACATGGCCTGCTGAGA  1587
GGAGCATGTCCTTAAAGCAGACCTTAACTCCCAGGGTAAACTGGCTATGTAACCCCCCA   1646
CCAAGGACTTGAGTTAAGGGAAGTGGTGAAAAAAAAAAAAAAAAAAAAAAAAAAA      1702
AAAA                                                          1706
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Continued Figure 2

b.

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ATGCCTAGCAGCATGTCAGGGTACATCCCGAGCTACTTAGATAAAGATGAGCTGTGTGTG      60
M  P  S  S  M  S  G  Y  I  P  S  Y  L  D  K  D  E  L  C  V
GTGTGTGGGGACAAGGCTACAGGATATCACTATAGATGCATCACCTGTGAGGGCTGCAAG     120
V  C  G  D  K  A  T  G  Y  H  Y  R  C  I  T  C  E  G  C  K
GGCTTTTTCCGGAGAACTATACAGAAAAATCTTCACCCTAGTTATTCATGTAAATATGAA     180
G  F  F  R  R  T  I  Q  K  N  L  H  P  S  Y  S  C  K  Y  E
GGAAAATGCGTGATAGACAAAGTAACCAGGAACCAGTGCCAAGAATGTCGCTTCAAA       237
G  K  C  V  I  D  K  V  T  R  N  Q  C  Q  E  C  R  F  K
AAGTGTATTGCGGTTGGCATGGCAACAGACCTGGTTTTGGATGACAGCAAGCGTCTG       294
K  C  I  A  V  G  M  A  T  D  L  V  L  D  D  S  K  R  L
GCAAAAAGAAGACTTATAGAAGAAAATCGAGAAAAGAGGCGCAAAGATGAAATACAG       351
A  K  R  R  L  I  E  E  N  R  E  K  R  R  K  D  E  I  Q
AAGTCGATGGTACAAAAACCCGAACCAACCCCGGAGGAGTGGGAGCTGATCCAGATT       408
K  S  M  V  Q  K  P  E  P  T  P  E  E  W  E  L  I  Q  I
GTCACCGAAGCTCACGTGACCACCAATGCACAGGGAAGTCACTGGAAGCAGAAACGG       465
V  T  E  A  H  V  T  T  N  A  Q  G  S  H  W  K  Q  K  R
AAATTTCTGCCAGAAGATATTGGGCAGGCTCCTATAGTTAATGCACCGGAGGGCGGAAAA   525
K  F  L  P  E  D  I  G  Q  A  P  I  V  N  A  P  E  G  G  K
GTGGATCTAGAAGCTTTCAGCCAGTTTACAAAAATTATCACCCCAGCAATTACAAGAGTG   585
V  D  L  E  A  F  S  Q  F  T  K  I  I  T  P  A  I  T  R  V
GTCGATTTTGCCAAAAAATTACCTATGTTTTGTGAGCTGCCATGTGAAGATCAGATCATC   645
V  D  F  A  K  K  L  P  M  F  C  E  L  P  C  E  D  Q  I  I
CTTCTTAAAGGCTGTTGCATGGAGATCATGTCGCTGCGAGCAGCAGTGCGCTACGACTCG   705
L  L  K  G  C  C  M  E  I  M  S  L  R  A  A  V  R  Y  D  S
GAAAGTGAGACCTTAACACTGAATGGGGAGATGGCAGTGACGCGAGGACAGCTAAAA      762
E  S  E  T  L  T  L  N  G  E  M  A  V  T  R  G  Q  L  K
AATGGAGGACTAGGAGTGGTATCTGACGCCATATTCGATTTGGGTGTTTCGCTGTCATCA   822
N  G  G  L  G  V  V  S  D  A  I  F  D  L  G  V  S  L  S  S
TTTAATCTTGACGATACGGAAGTCGCCTTGTTGCAATCTGTGCTCCTAATGTCATCAGAT   882
F  N  L  D  D  T  E  V  A  L  L  Q  S  V  L  L  M  S  S  D
CGTCCCGGTCTCTCCAGTGTGGAAAGAATAGAAAAGTGCCAAGAAGGTTTCCTCTTG      939
R  P  G  L  S  S  V  E  R  I  E  K  C  Q  E  G  F  L  L
GCTTTTGAACACTACATTAACTACAGGAAACACAACGTTGCACACTTTTGGCCAAAA       996
A  F  E  H  Y  I  N  Y  R  K  H  N  V  A  H  F  W  P  K
CTGCTGATGAAAGTCACCGACCTCCGCATGATTGGAGCCTGCCACGCCAGCAGGTTC      1053
L  L  M  K  V  T  D  L  R  M  I  G  A  C  H  A  S  R  F
TTGCACATGAAAGTGGAGTGCCCCACTGAACTGTTCCCCCCACTGTTCTTAGAAGTG      1110
L  H  M  K  V  E  C  P  T  E  L  F  P  P  L  F  L  E  V
TTTGAGGATTAGAAGAGACTGAGCTTCAGGATTCTCGGCACTACTGGGGTCTCCCCT      1167
F  E  D  -
TCCATTCCATTGCCTCCGTCACTTTTTTGTTCTCGTCCGTTCTGAATAGACATAGATGA    1226
AAATGTTCCTCTGATGCGGGTACTCGTGACTATCGTATTTTTGTTATTCTGTCCTTTTGA   1286
TGTGAAGTTTATCCGTGGAAGTTTATCCCCTTTTGTGCTGAAGTGGCTGTCGACACATT    1345
ACTCATCAGAGGGTTAGGCAGTCCTTCCAGTTATAAAGTGATTATTGTTTAAAAAAAA     1403
AAAAAAAAAAAAAAAAAA                                             1422
```

Figure 2 cDNA and deduced amino acid sequences of *Microhyla fissipes* thyroid hormone receptor gene. (a) *TRα*; (b) *TRβ*. The start codon (ATG) and the stop codon (TAG or TGA) are in bold.

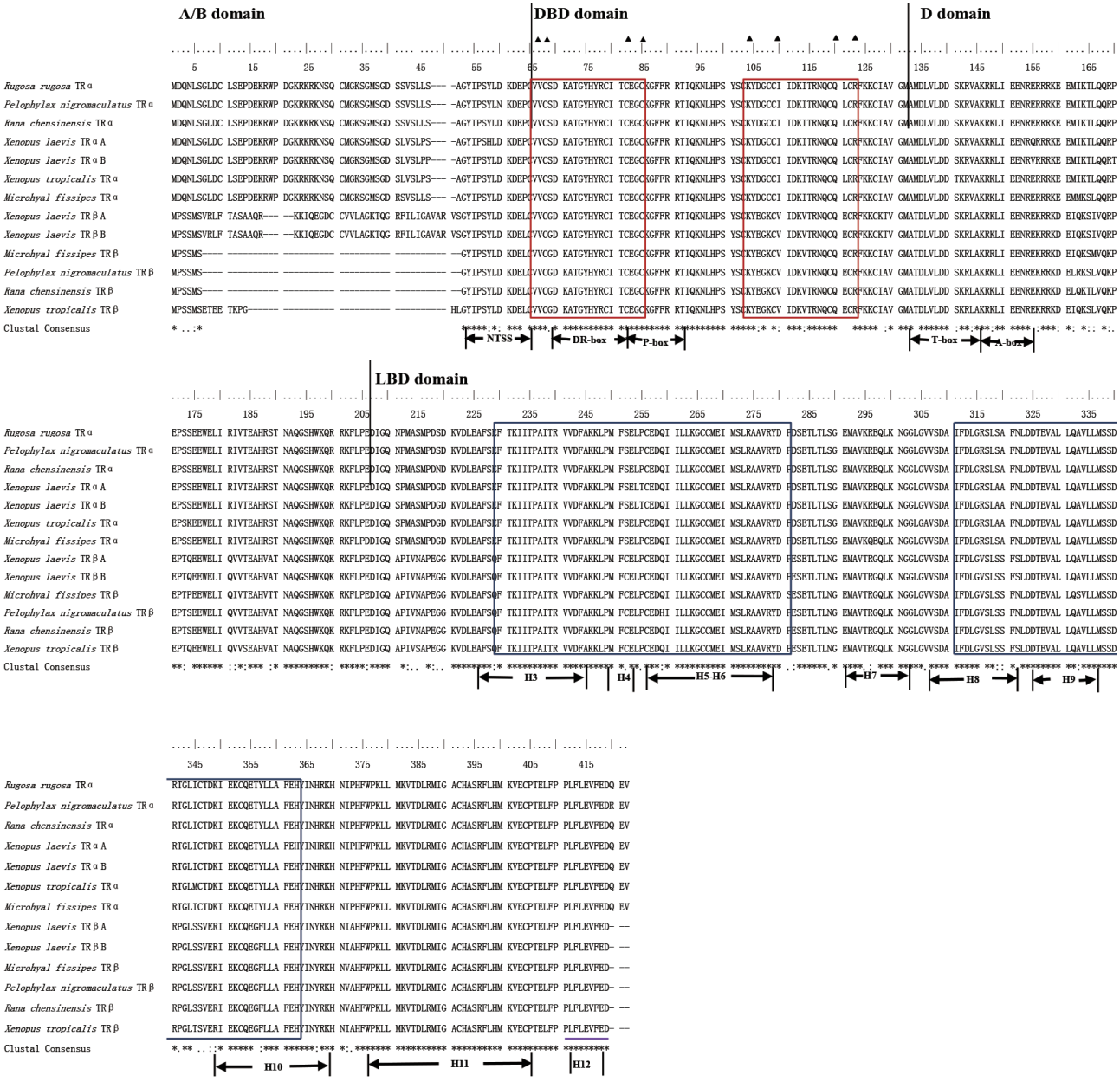

Figure 3 Alignments of TR amino acid sequences from *Microhyla fissipes* with other amphibian species. Asterisk (*) indicated conserved amino acids and hyphens (−) represented spaces inserted to maximize similarity. Colon (:) indicates conservation between groups of strongly similar properties, and period (.) indicates conservation between groups of weakly similar properties. The black vertical lines indicate the borders of four domains: the N-terminal hypervariable region (A/B domain), DNA-binding domain (DBD domain), hinge region (D domain) and ligand binding domain (LBD domain). Triangle indicated the conserved cysteine residues that comprise the two zinc finger with red box of the DBD. The conserved N-terminal signature sequence (NTSS), DR-box, P-box, T-box, A-box and Helices (H3-H12) are figured out. Motif I and Motif II are boxed by blue while activation domain (AF2-AD) is purple double underlined.

the morphology of *M. fissipes* tadpoles has changed dramatically, and the characteristic of morphology change trend was similar to that during the natural metamorphosis (data not shown). To determine whether exogenous T3 could potentially regulate the expression pattern of *TRα* and *TRβ*, we exposed the premetamorphic tadpoles to T3 and analyzed by qPCR. These results showed that *M. fissipes TRα* and *TRβ* mRNA expression were significantly increased by T3 at 12 h and 24 h, respectively. And then *TRα* and *TRβ* expression decreased to the lower level at 48 h than 0 h (Figure 5). Furthermore, the same gene expression pattern of *TRβ* has been detected between exogenous T3 induced and natural metamorphosis (Zhao *et al.*, 2016). Therefore, different responsiveness of *TRα* and *TRβ* to T3 indicated different functions of them in the metamorphosis.

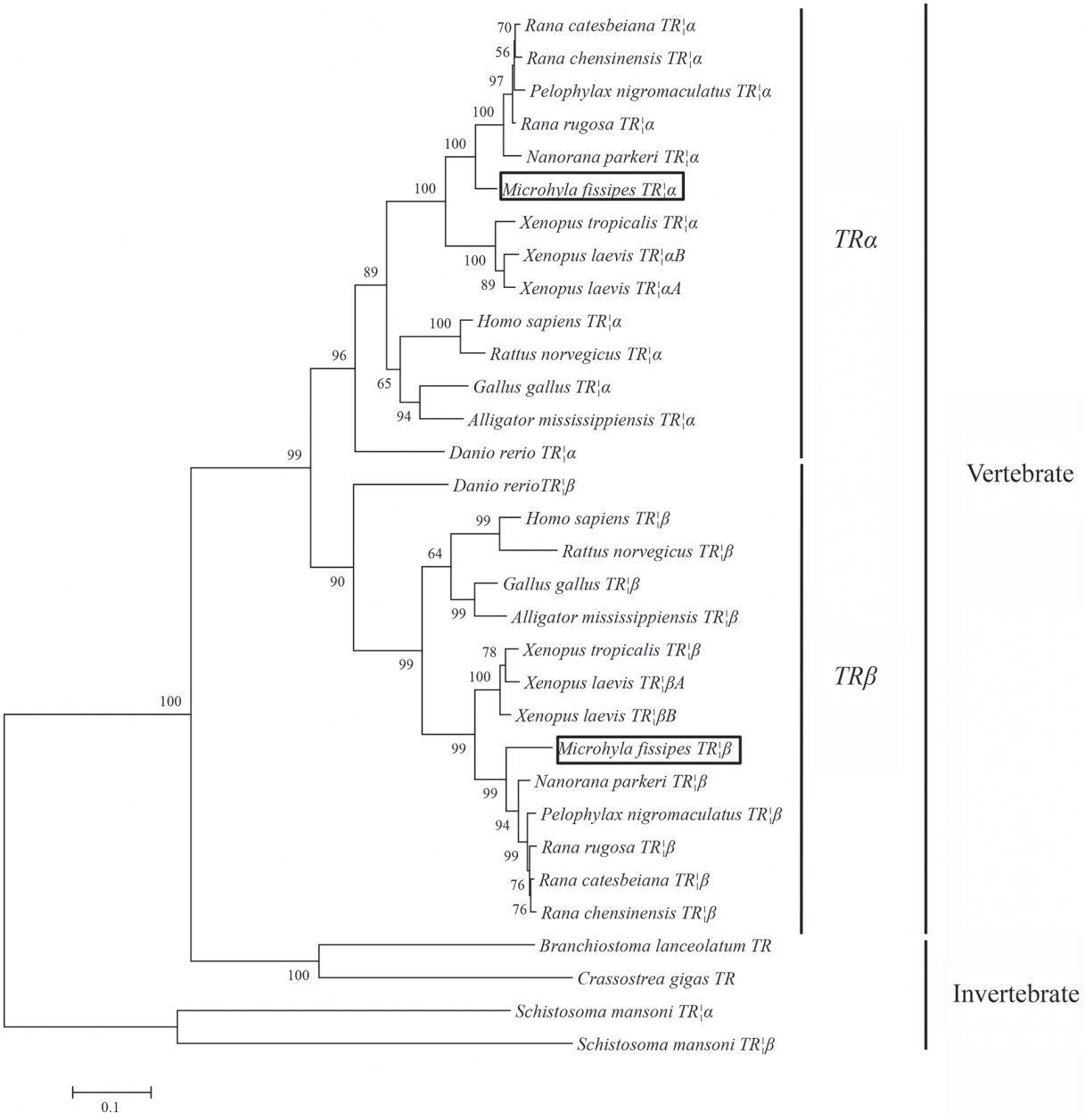

Figure 4 Phylogenetic tree of nucleotide sequences of *TRs* genes of different species using the the maximum likelihood method. Numbers at the branches represent the bootstrap support values. *Microhyla fissipes TRα* and *TRβ* are highlighted in the box, respectively.

4. Discussion

This study was designed to determine *TRα* and *TRβ* sequences and analyze their expression patterns after T3 exposure to gain further insights as to how these genes may function in metamorphosis. Therefore, *TRα* and *TRβ* genes of *M. fissipes* were cloned by RNA-seq and RACE. Phylogenetical analysis showed *M. fissipes TRα* and *TRβ* gene had high homology with the corresponding genes of other amphibians at both the nucleotide and the amino acid level, respectively, confirming their identities. The expression patterns after T3 treatment indicated the important roles of *TRα* and *TRβ* during the metamorphosis.

Both TRα and TRβ amino acid structure identified in *M. fissipes* possessed the typically functional domains of the NR superfamily. All of them had a conserved DBD domain with two zinc fingers, which determined the binding specificity of nuclear receptors (Natalia and Thorsten, 2004), and a LBD domain containing the typical 12 helices (Marchand *et al.*, 2001). The P-box determining DNA binding specificity interacted with

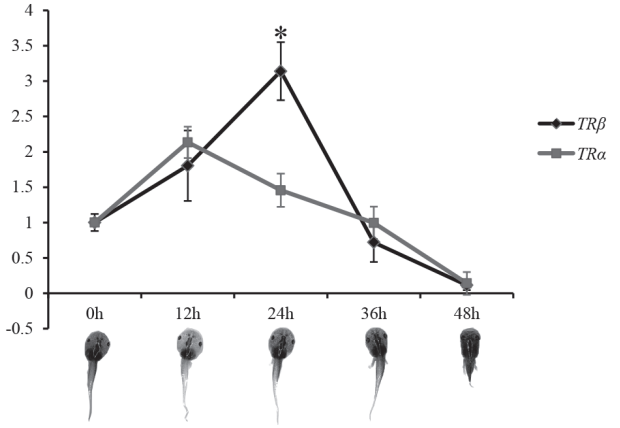

Figure 5 Relative expression levels of *TRs* genes after exposure to 10 nmol/L T3. Data were expressed as the mean fold difference (mean ± SD, $n = 3$) from the control group. Significant differences between treatment groups and the controls were indicated by * ($P < 0.05$).

the specific response element AGGTCA, and the T-box and A-box regions contributed to dimerization and DNA binding stabilization, respectively (Manchado *et al.*, 2009). The conserved NTSS (GYIPS(Y/H) L(D/N) KDE(P/L)) which was the TR specific motif was also detected in the C-terminus of the variable A/B domain of *M. fissipes* TRs (Wu *et al.*, 2007). The deletion of 42 amino acids in the A/B domain of TRβ indicated its different function from TRα. The conserved AF2-AD motif (LFLEVF) played an important role in recruiting a coactivator (Nagy *et al.*, 1999; Nelson and Habibi, 2009). The conservation of structure and functional roles of the above mentioned sites in TRs would be consolidated by their high identity throughout the evolution of vertebrates.

TRα and *TRβ* have been demonstrated to have high homology across vertebrates (Oka *et al.*, 2013). In this study, the amino acid and nucleotide sequence homologies of *TRα* and *TRβ* between *M. fissipes* and other amphibians were over 90%, but lower homology with invertebrates. In the phylogenetic tree, vertebrate *TRα* and *TRβ* were located in two clearly separated clades, in accordance with the fact that *TRα* and *TRβ* may be the products of an ancient gene duplication event during evolution (Chen *et al.*, 2014). Furthermore, the homologies of *TRα* and *TRβ* to the corresponding genes from other species accorded with their evolutionary relationship. Only one *TRα* and *TRβ* gene were identified in amphibian except in *X. laevis* which is tetraploid. The homologies of nucleotide sequences and deduced amino acid sequences between *TRα* and *TRβ* in *M. fissipes* were 61% and 72%, respectively. The homology of *TRα* to *TRβ* in *M. fissipes* was lower than *R. nigromaculata* (72% and 86%) and *X. laevis* (74% and 85%). Low sequence identification of *TRα* and *TRβ* may indicate their different regulated function in *M. fissipes* metamorphosis, which is also implied by their different expression pattern during natural metamorphosis (Zhao *et al.*, 2016).

Metamorphosis is a critical developmental stage mediated by TH in amphibian (Wang *et al.*, 2008). The function of *TRs* as transducers of TH responses has converted NRs in targets to clarify the molecular mechanisms that govern metamorphosis (Manchado *et al.*, 2009). To understand the potential importance of these two receptors in *M. fissipes* metamorphosis and function of exogenous ligand for the receptor systems, we examined their mRNA expression levels by qPCR after T3 exposure. T3 not only induced *M. fissipes* premetamorphic tadpoles metamorphosis at the morphology and histology level (data not shown), but also induced *TRα* and *TRβ* expression, which have also been reported in *X. laevis*, *X. troplis* and *R. catesbeiana* (Shi, 1999; Wang *et al.*, 2008). During the natural metamorphosis, *TRβ* expression increased dramatically and correlated with the endogenous THs, while *TRα* expression slightly increased; and all of them decreased at the end of metamorphosis (Shi, 1999; Zhao *et al.*, 2016). In this study, *TRα* expression reached peak at 12 h and then decreased from 12 h to 48 h. While dramatically up-regulated *TRβ* expression was observed after exposure of T3 within 24 h, and it was down-regulated from 24 h and with the lowest expression observed at 48 h of T3 treatment. These results suggested that *TRβ* expression pattern after T3 treatment is the same as that during natural metamorphosis, and the expression of *TRβ* in tadpoles treated with T3 for 24h also resembled its expression in the tadpoles at the climax of metamorphosis (Zhao *et al.*, 2016), which correlated with the morphological and histological results. Therefore, we can use T3 to simulate metamorphosis for further research on metamorphosis which will be high-efficiency and time saving. Moreover, *M. fissipes* could also serve as the model to assay environmental compounds on TH signaling disruption, while expression of *TRs* (in particular *TRβ*) has been also used as a molecular biomarker for assaying TH signaling disrupting actions in *X. laevis* and *R. nigromaculata* because of their response to TH (Opitz *et al.*, 2006; Veldhoen *et al.*, 2006; Lou *et al.*, 2014). Due to the specific expression level of *TRs* during *M. fissipes* natural metamorphosis and dramatically up-regulated *TRβ* mRNA expression after T3 treatment, we will use genome editing tools such as CRISPR/Cas9 to illustrate the mechanism of *TRα* and *TRβ* in *M. fissipes*. In conclusion, *TRα* and *TRβ* from *M. fissipes* were cloned

and characterized for the first time. Functional site and phylogenetic analysis indicated the conserved function of *TRs* from invertebrate to vertebrates. Premetamorphic tadpoles treated with T3 for 24 h resembled the climax of metamorphosis tadpoles during natural metamorphosis, and *TRβ* mRNA expression analysis could serve as a sensitive molecular testing approach to study effects of environmental compounds on the thyroid system in *M. fissipes*.

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